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Multidrug-Resistant *Candida auris* Misidentified as *Candida haemulonii*: Characterization by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry and DNA Sequencing and Its Antifungal Susceptibility Profile Variability by Vitek 2, CLSI Broth Microdilution, and Etest Method

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***Candida auris* is a multidrug-resistant yeast that causes a wide spectrum of infections, especially in intensive care settings. We investigated *C. auris* prevalence among 102 clinical isolates previously identified as *Candida haemulonii* or *Candida famata* by the Vitek 2 system. Internal transcribed spacer region (ITS) sequencing confirmed 88.2% of the isolates as *C. auris*, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) easily separated all related species, *viz.*, *C. auris* ($n = 90$), *C. haemulonii* ($n = 6$), *C. haemulonii* var. *vulnera* ($n = 1$), and *Candida duobushaemulonii* ($n = 5$). The *in vitro* antifungal susceptibility was determined using CLSI broth microdilution (CLSI-BMD), the Vitek 2 antifungal susceptibility test, and the Etest method. *C. auris* isolates revealed uniformly elevated fluconazole MICs (MIC_{50}, 64 μg/ml), and an alarming percentage of isolates (37%) exhibited elevated caspofungin MICs by CLSI-BMD. Notably, 34% of *C. auris* isolates had coexisting elevated MICs (≥2 μg/ml) for both fluconazole and voriconazole, and 10% of the isolates had elevated coexisting MICs (≥2 μg/ml) to two additional azoles, i.e., posaconazole and isavuconazole. In contrast to reduced amphotericin B MICs by CLSI-BMD (MIC_{50}, 1 μg/ml) for *C. auris*, elevated MICs were noted by Vitek 2 (MIC_{50}, 8 μg/ml), which were statistically significant. *Candida auris* remains an unnoticed pathogen in routine microbiology laboratories, as 90% of the isolates characterized by commercial identification systems are misidentified as *C. haemulonii*. MALDI-TOF MS proved to be a more robust diagnostic technique for rapid identification of *C. auris*. Considering that misleading elevated MICs of amphotericin B by the Vitek AST-YS07 card may lead to the selection of inappropriate therapy, a cautionary approach is recommended for laboratories relying on commercial systems for identification and antifungal susceptibility testing of rare yeasts.**

In recent years, two species, namely, *Candida pseudohaemulonii* and *Candida auris*, which are phylogenetically closely related to *Candida haemulonii* in the Metschnikowiaceae clade, have been described (1). The yeast *C. auris*, isolated from the external ear canal of a Japanese patient, was described as a new species in 2009 (2). This pathogen was recently recognized as an emerging multidrug-resistant (MDR) yeast that can cause a wide spectrum of infections, ranging from fungemia to deep-seated infections, especially in intensive care settings (3–8). *Candida auris* is reported to be misidentified as *C. haemulonii*, *Candida famata*, and *Rhodotorula glutinis* by commercial identification systems, such as Vitek 2 and API20C-AUX, and exhibits a unique susceptibility profile (5–8). Notably, the potential of clonal transmission of *C. auris*, highly elevated MICs to fluconazole, and reduced susceptibility to voriconazole, caspofungin, and flucytosine are matters of serious concern (7–9). Therefore, accurate identification is important, because treatment strategies are often directed by species characterization of *Candida*. Further, a recent report of misleadingly high MICs of amphotericin B and caspofungin using the commercially available Vitek 2 automated system in a patient with pericarditis due to *C. auris* highlights issues of inappropriate treatment strategies if accurate susceptibility testing is not available (4). In the present era, molecular techniques and a growing database of fungal genome sequences have facilitated the reliable identification of phylogenetically related and phenotypically identical species. Herein, we investigated the prevalence of *C. auris* in a collection of clinical isolates previously phenotypically identified as *C. haemulonii*/*C. famata*, using internal transcribed spacer region (ITS) sequencing and evaluated matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for rapid and reliable identification of this yeast. Further, their *in vitro* antifungal susceptibilities to the 10 antifungals were determined using the CLSI broth microdilution (CLSI-BMD) method,

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and data obtained by CLSI were compared with those obtained by the commercial Vitek 2 system and the Etest method.

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MATERIALS AND METHODS

Isolates. A total of 102 clinical isolates, from individual patients, previously identified as *Candida haemulonii*/*C. famata* by the Vitek 2 compact system (bioMérieux, Marcy l'Etoile, France) in 4 tertiary care hospitals in Delhi, North India, and a single center in Kochi, Kerala, South India, from 2010 to 2014 were included. Three centers were about 1,000-bed general hospitals, and the remaining 2 were a pediatric hospital and a transplant center. The pediatric hospital had neonatal intensive care units and surgical intensive care facilities. The isolates were mainly from patients with candidemia (blood; $n = 78$), and other specimens from invasive *Candida* infections included gangrenous tissue ($n = 4$), pleural fluid ($n = 6$), and peritoneal fluid ($n = 7$). Also, 7 isolates from urine ($n = 4$) and sputum ($n = 3$) specimens from immunocompromised patients were included. The control and type strains of three *C. auris* isolates from Korea (KCTC 17809, KCTC 17810) and Japan (DSM 21092^T) and one isolate each of *C. haemulonii* strain CBS 7802 and *C. duobushaemulonii* strain CBS 7798^T were also analyzed.

Phenotypic characterization. The isolates were identified by standard mycological procedures, including colony color on CHROMagar *Candida* medium (Difco, Becton Dickinson & Company, Baltimore, MD, USA) and morphology on rice Tween 80 agar. Growth patterns at different temperatures, 37°C, 42°C, and 45°C, were also observed (1). Additionally, the assimilation profile of all yeast isolates was done by commercially available API strips (ID32C; bioMérieux, Marcy l'Etoile, France), which were read and interpreted at 48 h.

Sequencing of ITS region. Genomic DNA was extracted from all test isolates along with reference strains as described by Xu et al. (11). DNA was amplified and sequenced using the ITS-1 (5′-TCCGTAGGTGAACC TTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) primers, which amplify the ITS region of the ribosomal subunit (8). Sequences were aligned, and GenBank Basic Local Alignment Search Tool (BLAST) searches were performed for species identification. For phylogenetic analyses, the ITS gene sequences of the *C. auris*, *C. haemulonii*, and *C. duobushaemulonii* isolates were aligned with the ClustalW program (version 1.82), and the final alignments were edited manually. A neighbor-joining (NJ) tree based on ITS gene sequences using 2,000 bootstrap replications was generated using MEGA version 5 (12). The sequences of the reference/type strains of *C. auris* from Japan (JCM 15448^T) and Korea (KCTC 17809 and KCTC 17810), along with *C. haemulonii* (CBS 5150, Portugal; CBS 7801, United States), *C. haemulonii* var. *vulnera* (CNMCL-7462, Spain), and *C. duobushaemulonii* (CBS 7799, USA), were retrieved from GenBank and included for the analysis.

MALDI-TOF MS. The ethanol-formic acid extraction procedure was followed according to the manufacturer's protocol for the identification of yeast isolates (13). The spectra were analyzed using the Flex Control 3.1 software (Bruker Daltonics, Inc., Billerica, MA, USA) and MALDI Biotyper OC version 3.1 (Bruker Daltonics, Bremen, Germany). Score values were analyzed as per manufacturer recommendations: a score of ≥2 indicated confidence to the species level, 1.7 to 1.99 indicated confidence to the genus level, and <1.7 indicated no identification.

MALDI data analysis. The MALDI Biotyper version 3 database contains spectra of 3 strains of *C. auris*, two from Korea (KCTC 17809 and KCTC 17810) and a type strain from Japan (DSM 21092^T). For phylogenetic analysis, spectra of 90 *C. auris* isolates were added manually to the library for the creation of a score-oriented dendrogram in Biotyper as described previously for *Aspergillus* species (14). The mass spectra of each quadruplicate of the respective isolates with a score value of >2 were considered for dendrogram preparation. Additionally, available spectra of reference strains of *C. auris* from Japan (DSM 21092^T) and Korea (KCTC 17809 and KCTC 17810) and of *C. haemulonii* (CBS 5149^T and CBS 5150), *C. duobushaemulonii* (CBS 7799 and CBS 7800), and *C. pseudohaemulonii* (CBS 10004 and CBS 12453^T) in the database were imported in the software for the analysis of the dendrogram. The dendrogram was generated by using the respective functionality of the MALDI Biotyper 3.1 offline client. The spectra of all the isolates tested were analyzed by a score-oriented dendrogram using an arbitrary distance level of 1,000 as the cutoff.

AST. (i) CLSI-BMD method. Antifungal susceptibility testing (AST) was carried out using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI-BMD), following the M27-A3 guidelines (15). Antifungals tested were amphotericin B (AMB; Sigma, St. Louis, MO, USA), fluconazole (FLU; Pfizer, Groton, CT, USA), itraconazole (ITC; Lee Pharma, Hyderabad, India), voriconazole (VRC; Pfizer), posaconazole (POS; Merck, Whitehouse Station, NJ, USA), isavuconazole (ISA; Basilea Pharmaceutica, Basel, Switzerland), flucytosine (5-FC; Sigma), caspofungin (CAS; Merck), micafungin (MFG; Astellas, Toyama, Japan), and anidulafungin (AFG; Pfizer). RPMI 1640 medium with glutamine without bicarbonate (Sigma) buffered to pH 7 with 0.165 mol/liter 3-*N*-morpholinepropanesulfonic acid (MOPS; Sigma) was used. Drug-free and yeast-free controls were included, and microtiter plates were incubated at 35°C and read visually after 24 h, as validated recently by Pfaller et al. (16, 17). CLSI-recommended *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains, and two reference strains of *C. auris* (KCTC 17809 and DSM 21092^T) were also included. Excepting AMB, the MIC endpoints for all the antifungals were defined as the lowest drug concentration that caused 50% growth inhibition *vis-à-vis* the drug-free controls. The MIC for AMB was defined as the lowest concentration at which there was 100% inhibition of growth. The susceptibility for all the isolates was performed by two different personnel on two occasions, which revealed reproducible results.

(ii) Vitek 2 Compact system using an AST-YS07 card. Susceptibility was determined using an AST-YS07 card, which tests the MIC of 6 antifungals, i.e., FLU, 5-FC, VRC, AMB, CAS, and MFG. All the *C. auris* isolates were tested as per the manufacturer's instructions. The time of incubation ranged from 18 to 27 h, based on the rate of growth in the drug-free control well, and the results were expressed as MICs in micrograms per milliliter.

(iii) Etest method. Further, the isolates which revealed >2-fold discrepancies in the antifungal MICs by the above-described two methods were also tested for susceptibility by Etest using *Etest Technical Guide 4*: *Antifungal Susceptibility of Yeasts* (AB Biodisk, bioMérieux, Solna, Sweden), as described previously (19, 20). The antifungals tested were AMB, CAS, and VRC. Briefly, the inoculum density of 0.5×10^3 to 2.5×10^3 cells/ml prepared for the CLSI-BMD test was used, and the test medium included RPMI 1640 with 1.5% agar supplemented with glucose (2%) and was buffered to pH 7.0 with MOPS. In addition, AMB was also tested on antibiotic medium 3 (AM3) agar plates. The plates were inoculated by dipping a sterile cotton swab into the inoculum and streaking it across the entire surface of the agar in three directions. The plates were dried for 15 min before the Etest strips (AB Biodisk) were applied and kept at 35°C and read visually after 24 h. The Etest MIC was defined as the drug concentration at which the border of the elliptical zone of complete inhibition intersected the scale on the antifungal test strip.

Statistical analysis. Statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA). MIC values from the CLSI-BMD, Vitek 2, and Etest methods were assessed by using the Student *t* test (paired sample). The Etest MIC endpoints, which were in between the 2-fold dilution scale of the CLSI method, were rounded to the corresponding next upper 2-log dilution to simplify comparisons. The discrepancies among MIC endpoints of ±2 dilutions (two wells) were used to calculate the essential agreement (EA).

***FKS* gene sequencing.** *Candida auris* isolates with elevated CLSI-BMD MICs of CAS (MICs ≥ 1 μg/ml) were subjected to sequencing of the *FKS1* and *FKS2* genes. Considering that the genome sequence of *C. auris* is not yet available, the published mutations in the *FKS* gene of echinocandin-resistant *Candida glabrata* isolates were used to analyze the mutations in

the *FKS* gene of *C. auris* isolates by sequence homology (21, 22). Genomic DNA was amplified and sequenced for hot spot regions of both the genes. The primers were designed based on the *C. glabrata FKS1* and *FKS2* gene sequences (GenBank accession no. XM_446406 and XM_448401, respectively). The primers for *FKS1* (FKS1HSF, 5′-CATTGCTATTTTTCTCAG TCATGC-3′; FKS1HSR, 5′-CCAACGGAAAAGACAGTGTTGA-3′) and *FKS2* (FKS2HSF, 5′-CTGTGACATTTTTCATTGCTG-3′; FKS2HSR, 5′-TCCAAGGAGTTAAGATGGAAATACC-3′) were designed using Primer3 software version 4 (http://primer3.ut.ee/). DNA sequences were analyzed with Sequencing Analysis software version 5.3.1 (Applied Biosystems). Consensus sequences were made using BioEdit software (version 7.0.5.3) and were aligned with hot spot *FKS* regions of reference *C. glabrata* (GenBank accession no. HM366439 for *FKS1* and HM366442 for *FKS2*).

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in GenBank under accession no. KF689009 to KF689022, KC692039 to KC692052, and KP862745 to KP862818.

RESULTS

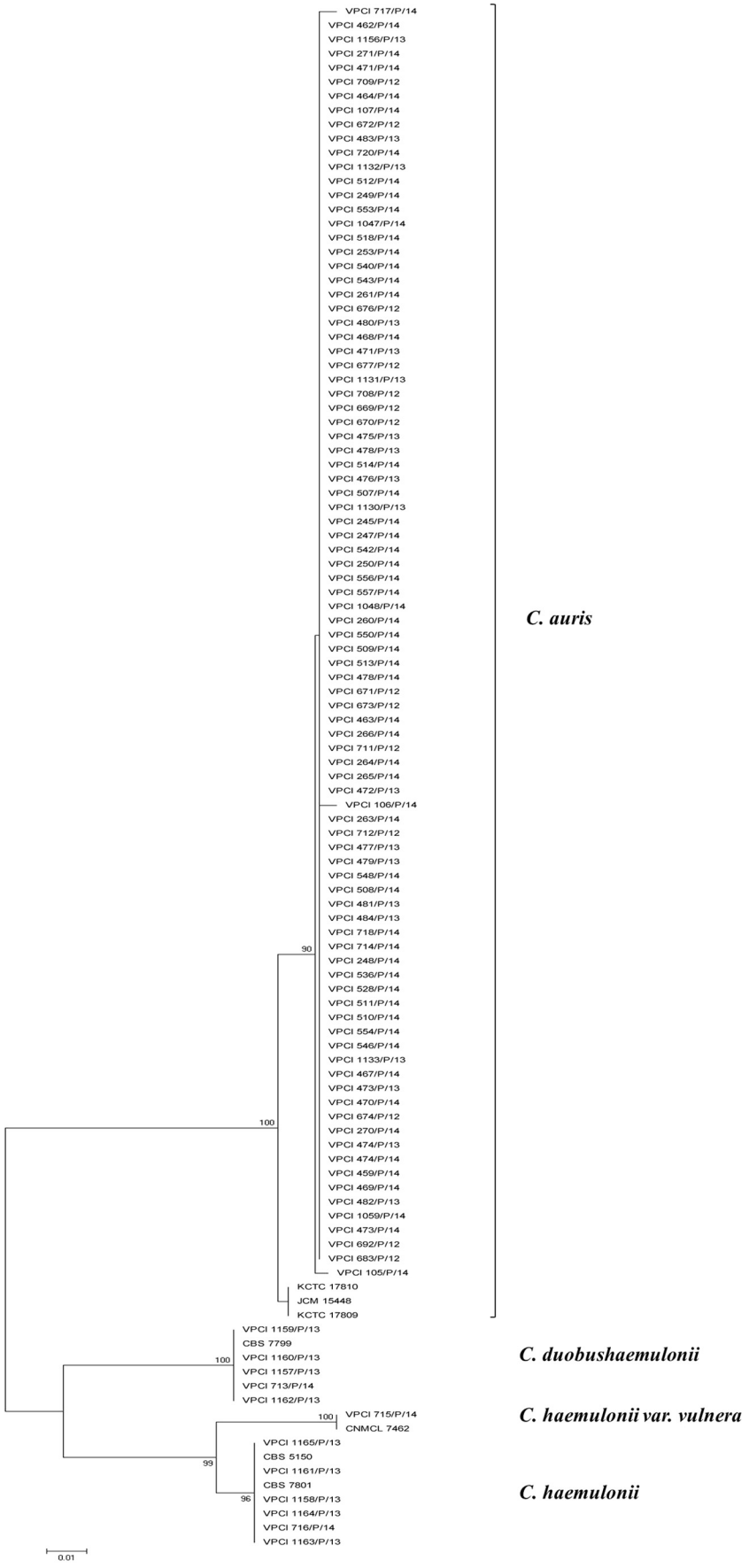
Vitek 2 identified 100 isolates as *C. haemulonii* (88 with 91 to 98% identity and 12 with a low discrimination profile), whereas the remaining 2 isolates were identified as *C. famata* (93% identity). Of the 102 isolates, 88.2% ($n = 90$) were confirmed as *C. auris* by ITS sequencing. The remaining 12 isolates were identified as *C. haemulonii* ($n = 6$), *C. haemulonii* var. *vulnera* ($n = 1$), and *C. duobushaemulonii* ($n = 5$). All *C. auris* ($n = 90$) isolates showed smooth, white to cream-colored colonies on Sabouraud dextrose agar (SDA), whereas they developed a pink color on CHROMagar *Candida* medium. Microscopic examination showed ovoid to elongated budding yeast cells occurring singly or in pairs. No pseudohyphae were formed on rice Tween 80 agar after 4 to 8 days of incubation at 28°C. They grew well at 37°C and 42°C. In contrast, *C. haemulonii* and *C. duobushaemulonii* isolates revealed pseudohyphae with blastoconidia and did not grow at 42°C. All the *C. auris* isolates were positive for assimilation of *N*-acetylglucosamine, succinate, and gluconate, whereas negative results were recorded for *C. haemulonii* and *C. duobushaemulonii*.

ITS sequences of the 90 isolates (GenBank accession no. KF689009 to KF689022, KC692039 to KC692052, KP862745 to KP862805, and KP862818) showed 99% homology (query coverage ranging from 98 to 100%) with *C. auris* isolates in GenBank (accession no. HE797773 and AB375772). However, the ITS sequences of 6 isolates (GenBank accession no. KP862806 to KP862811) showed 100% homology with *C. haemulonii* (GenBank accession no. KM014586 and JX459689), and 5 isolates (GenBank accession no. KP862813 to KP862817) showed 100% homology with *C. duobushaemulonii* (GenBank accession no. KM361511 and KJ476202). Also, a solitary isolate (VPCI 715/P/14; GenBank accession no. KP862812) showed 100% homology with *C. haemulonii* var. *vulnera* (GenBank accession no. JX459686). The ITS tree yielded 3 distinct clades with a good bootstrap value (99%) and enabled the differentiation of *C. auris* from *C. haemulonii* and *C. duobushaemulonii* strains (Fig. 1). All the Indian *C. auris* ($n = 90$) strains exhibited 99 to 100% sequence similarity among themselves. However, *C. auris* isolates from Japan and Korea formed a separate group away from Indian *C. auris* isolates in the same clade. The other species of the *C. haemulonii* complex were well differentiated in 2 separate clades. *Candida duobushaemulonii* ($n = 5$) formed clade 2, whereas *Candida haemulonii* ($n = 6$) and *C. haemulonii* var. *vulnera* ($n = 1$) formed two groups in clade 3.

MALDI-TOF MS. The MALDI-TOF mass spectra obtained for all *C. auris* isolates were evaluated against the original MALDI Biotyper OC version 3.1 database. The mean MALDI-TOF MS score of the tested *C. auris* isolates was 2.167 (range, 2.013 to 2.347). Of the 90 *C. auris* isolates, the majority ($n = 77$, 85.5%) were identified to the species level at the first attempt with a score value of >2. The remaining isolates ($n = 13$) were also identified as *C. auris* but with a score value of <2 and >1.7. These isolates were repeated and revealed high score values in the second attempt. All the *C. haemulonii* ($n = 6$) and *C. duobushaemulonii* ($n = 5$) strains were identified to the species level (score value, >2). Further, a solitary isolate of *C. haemulonii* was also identified to the variety level with a score value of >2 (*C. haemulonii* var. *vulnera*). The dendrogram clearly revealed separation of members of the Metschnikowiaceae clade in 4 phylogroups (Fig. 2). The mass spectra of the Indian *C. auris* isolates showed marked similarity, whereas the Japanese ($n = 1$) and Korean *C. auris* isolates ($n = 2$) exhibited variations in mass spectra among themselves and with those of Indian *C. auris* isolates, resulting in a separate cluster in *C. auris* (phylogroup 4). The dendrogram generated was in agreement with the phylogenetic NJ tree with ITS sequences.

***In vitro* susceptibility and *FKS* mutation analysis.** The *in vitro* susceptibility data and the MIC distribution of *C. auris* isolates using different methods along with essential agreements between the tested methods are presented in Tables 1 and 2.

CLSI-BMD. FLU exhibited no activity against 89% ($n = 80$) of *C. auris* isolates (MIC of 16 to >64 μg/ml), whereas the remaining 10 isolates revealed a MIC of 4 μg/ml. Similarly, an elevated MIC_{90}, i.e., 8 μg/ml, was noted for VRC. Notably, 58% of *C. auris* isolates ($n = 52$) showed VRC MICs of ≥1 μg/ml. In contrast, MIC_{50} values of POS (0.06 μg/ml) and ISA (0.25 μg/ml) were relatively low compared to that of VRC (Table 1). Also, 11% of *C. auris* isolates revealed MICs of ≥1 μg/ml for both POS and ISA, and a solitary isolate showed a MIC of ≥1 μg/ml only to ISA. All *C. auris* isolates showed reduced MICs to ITC (geometric mean [GM] MIC, 0.15 μg/ml). Furthermore, *C. auris* isolates had AMB MIC_{50} values of 1 μg/ml; however, 15.5% ($n = 14$) of the isolates revealed MICs of ≥2 μg/ml for AMB. Moreover, elevated GM MICs were observed for CAS (0.58 μg/ml) in comparison to MFG (0.11 μg/ml) and AFG (0.23 μg/ml). Notably, 37% ($n = 33$) of the *C. auris* isolates revealed MICs of ≥1 μg/ml to CAS. Also, all the echinocandins had no activity in 8% ($n = 7$) of the isolates, with MICs ranging from 4 to >8 μg/ml (Table 2). Further, 88% of *C. auris* isolates had reduced MICs to 5-FC (GM MIC, 0.4 μg/ml), whereas 11 isolates showed highly elevated MICs (≥32 μg/ml). In contrast to *C. auris*, all the *C. haemulonii* ($n = 7$) and *C. duobushaemulonii* ($n = 5$) isolates had markedly elevated AMB MICs ranging from 4 to 16 μg/ml. Also, variable FLU MICs were observed for *C. haemulonii* (MIC range, 2 to >64 μg/ml) and *C. duobushaemulonii* (MIC range, 1 to 16 μg/ml). However, reduced MICs of VRC (MIC range, 0.03 to 0.5 μg/ml) were noted for both the *C. haemulonii* and *C. duobushaemulonii* isolates, except a solitary isolate of *C. haemulonii*, which showed a MIC of 4 μg/ml. Moreover, both the *C. haemulonii* and *C. duobushaemulonii* isolates exhibited reduced GM MICs to ISA (0.027 μg/ml and 0.023 μg/ml), followed by POS (0.05 μg/ml and 0.11 μg/ml) and ITC (0.31 μg/ml). Also, in contrast to *C. auris* (GM MIC, 0.58 μg/ml), both *C. haemulonii* (GM MIC, 0.19 μg/ml) and *C. duobushaemulonii* (GM MIC, 0.14 μg/ml) showed reduced MICs to CAS. However, a wide MIC range (<0.125 to 64 μg/ml) for 5-FC was observed for *C. haemulonii*, while reduced MICs (GM MIC, 0.125 μg/ml) were found for *C. duobushaemulonii* (Table 1).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C. haemulonii var. vulnera
C. haemulonii

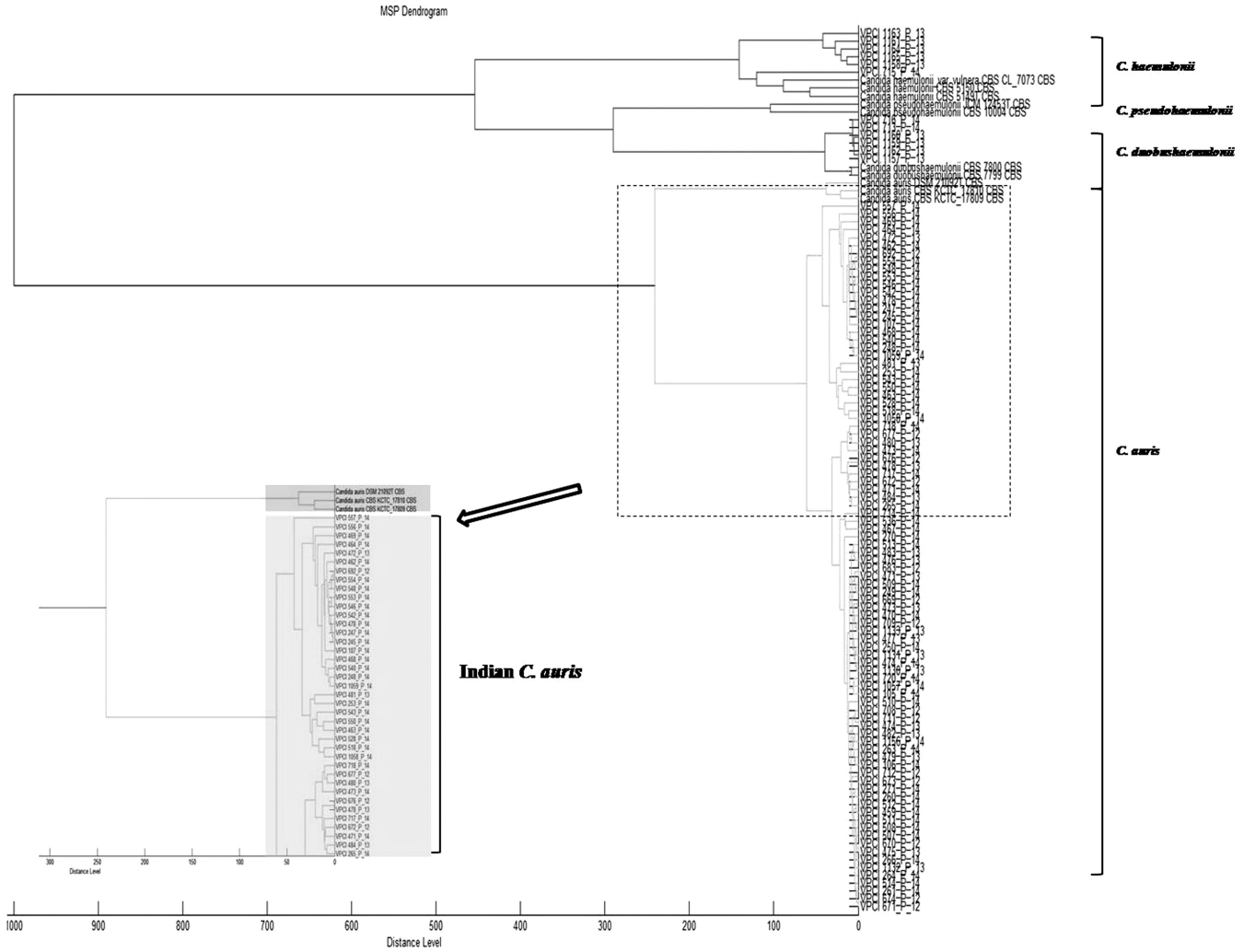

FIG 2 Score-oriented dendrogram of the main spectra (MALDI Biotyper 3.1; Bruker Daltonics) by using average linkages clustering the MALDI-TOF spectra of Indian *Candida auris* ($n = 90$) along with the *C. haemulonii* ($n = 6$), *C. duobushaemulonii* ($n = 5$), and *C. haemulonii* var. *vulnera* ($n = 1$) isolates. *Candida auris* (DSM 21092^T, KCTC-17809, and KCTC-17810) and *C. pseudohaemulonii* (JCM 12453^T and CBS 10004), *C. haemulonii* (CBS 5150 and CBS 5149^T), *C. haemulonii* var. *vulnera* (CL-7073), and *C. duobushaemulonii* (CBS 7800 and CBS 7799) were added to make the clustering in MALDI-TOF MS more robust. The isolates were classified into 4 phylogroups. The inset depicts the variation among Indian, Japanese, and Korean *C. auris* isolates leading to two clusters in phylogroup 4.

***FKS* gene sequencing of *C. auris* isolates with elevated caspofungin MICs (≥1 μg/ml).** Amplification of *FKS1* and *FKS2* regions generated amplicons of 391 bp and 460 bp, respectively. Mutations reported for caspofungin-resistant *C. glabrata* were not observed in the *FKS1* and *FKS2* regions of any of the tested *C. auris* strains.

Vitek 2. In contrast to low AMB MICs recorded by CLSI for *C. auris*, exceptionally elevated AMB MICs (CLSI MIC_{50} of 1 μg/ml compared to Vitek MIC_{50} of 8 μg/ml) were noted, which were statistically significant ($P < 0.0001$). Barring a solitary isolate of *C. auris*, all the other isolates had MICs of ≥8 μg/ml for AMB (Table 2). Also, the AMB MICs of *C. haemulonii* and *C. duobushaemulonii* ranged from 8 to 16 μg/ml, which was in concordance with CLSI. Vitek 2 MIC_{50} values of VRC (1 μg/ml), CAS (0.5 μg/ml), and MFG (0.125 μg/ml) of *C. auris* isolates were in 100% agreement with those by the CLSI method. Vitek 2 MIC_{50} values of FLU (32 μg/ml) and 5-FC (1 μg/ml) were within ±2 dilutions by CLSI-BMD.

Etest. Similar to CLSI MICs, low MIC_{50} values of AMB for *C. auris* isolates were observed by Etest on AM3 medium (0.5 μg/ml) and on RPMI agar (1 μg/ml). Except a solitary isolate, all *C. auris* isolates showed MICs of ≤1 μg/ml for AMB. The MIC_{50} (1 μg/

FIG 1 Phylogenetic tree based on partial ITS sequences of Indian *C. auris* ($n = 90$), *C. duobushaemulonii* ($n = 5$), *C. haemulonii* ($n = 6$), and *C. haemulonii* var. *vulnera* ($n = 1$) isolates using neighbor-joining analysis with 2,000 bootstrap replications. Sequences of reference strains of *Candida haemulonii* (CBS 5150, Portugal; CBS 7801, United States), *C. duobushaemulonii* (CBS 7799, United States), and *C. haemulonii* var. *vulnera* (CNMCL-7462, Spain) along with Japanese (JCM 15448^T) and Korean (KCTC-17809 and KCTC-17810) *C. auris* isolates were retrieved from GenBank for the analysis. Bootstrap values are shown above the branches.

TABLE 1 *In vitro* antifungal susceptibility profile of *C. auris*, *C. haemulonii*, and *C. duobushaemulonii* strains by the CLSI M27-A3 broth microdilution method

Species tested	MIC parameter	MIC (μg/ml)[a]									
		AMB	ITC	VRC	ISA	POS	FLU	5-FC	CAS	MFG	AFG
C. auris ($n = 90$ isolates)	GM	0.8	0.15	1.01	0.18	0.06	36	0.5	0.58	0.11	0.23
	MIC_{50}	1	0.125	1	0.25	0.06	64	0.25	0.5	0.125	0.125
	MIC_{90}	4	0.5	8	2	2	64	8	1	0.25	0.5
	MIC range	0.125–8	<0.03–2	<0.03–16	<0.015–4	<0.015–8	4–>64	<0.125– >64	0.125–8	<0.015–8	<0.015–8
C. haemulonii ($n = 7$ isolates)	GM	12.7	0.314	0.31	0.027	0.05	32	2.82	0.19	0.28	0.44
	MIC_{50}	16	0.25	0.5	0.015	0.125	64	32	0.25	0.25	0.5
	MIC_{90}	16	0.5	2	0.25	0.25	64	64	1	1	1
	MIC range	4–16	0.25–0.5	<0.03–4	<0.015–0.5	0.015–0.25	2–>64	0.125–>64	0.06–1	0.125–1	0.25–1
C. duobushaemulonii ($n = 5$ isolates)	GM	16	0.315	0.068	0.023	0.11	6.35	0.125	0.14	0.35	0.56
	MIC_{50}	16	0.25	0.06	0.015	0.125	8	0.125	0.125	0.5	0.5
	MIC_{90}	16	0.5	0.125	0.06	0.25	16	0.125	0.25	1	1
	MIC range	>16	0.25–0.5	<0.03–0.125	<0.015–0.06	0.03–0.125	1–16	<0.125	0.06–0.25	0.125–1	0.5–1

[a] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; FLU, fluconazole; 5-FC, 5-flucytosine; CAS, caspofungin; MFG, micafungin; AFG, anidulafungin.

ml) of VRC was similar to that by CLSI-BMD. In contrast, Etest CAS MICs were better differentiated than CLSI-BMD MICs, and Vitek showed a wide range from 0.002 to 4 μg/ml. Specifically, 33 *C. auris* isolates which showed MICs of ≥1 μg/ml for CAS by CLSI revealed highly variable MICs ranging from 0.064 to 4 μg/ml by Etest. Interestingly, 26 of these 33 isolates revealed MICs of ≤1 μg/ml for CAS by Vitek. The remaining 7 isolates had MICs of 4 μg/ml, which were in agreement with the CLSI-BMD MICs. Further, Etest MICs of *C. haemulonii* and *C. duobushaemulonii* for AMB, CAS, and VRC were within ±2 dilutions of CLSI MICs.

Agreement between methods. The essential agreement within ±2 dilutions for the comparison of 24-hour CLSI-BMD with Vitek 2 and Etest results showed 10% and 81% for AMB, 90% and 48% for CAS, and 91% and 79% for VRC, respectively.

DISCUSSION

The present study highlights that *Candida auris* remains an unnoticed pathogen in routine microbiology laboratories in India, as 90% of the isolates characterized by commercial identification systems misidentify this yeast as *C. haemulonii*. In the past 5 years, *Candida auris* has emerged as a significant pathogen in tertiary care general hospitals and a pediatric center in north and south India, representing 8.6% to 30% of cases of candidemia (7, 8). The actual prevalence of *C. auris* in varied clinical settings in India is unexplored, as the majority of centers do not perform molecular or MALDI-TOF MS-based identification. In this work, a large number of *C. auris* isolates were tested for antifungal susceptibility with three methods which showed uniform fluconazole resistance and an alarming percentage of isolates (37%) exhibiting elevated caspofungin MICs by CLSI-BMD. Taken together, 10% of isolates showed highly elevated MICs to 4 antifungals drugs (AMB, FLU, CAS, VRC) by the CLSI-BMD method. Notably, 34% of isolates had coexisting elevated MICs for two commonly used azoles, i.e., FLU and VRC (MICs of ≥2 μg/ml), and 10% of the isolates had elevated coexisting MICs (≥1 μg/ml) to two additional azoles, i.e., POS and ISA. Considering the frequent prevalence of MDR strains of *C. auris* in the intensive care units and other wards of 5 different hospitals in the present series, the accurate identification and antifungal susceptibility testing of this yeast is pertinent for guiding therapy and determining the prognosis in such settings. Also, accurate identification of the cryptic species *C. auris* is important in assessing the epidemiology and pathogenicity of the disease

TABLE 2 Distribution of MICs of amphotericin B, caspofungin, and voriconazole obtained by 3 different methods for *Candida auris* ($n = 90$) strains

Drug tested[a]	Test method	No. of isolates at MIC (μg/ml)												MIC (μg/ml)	
		<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	>16	MIC_{50}	MIC_{90}
AMB	CLSI-BMD				2	16	23	35	4	6	4			1	4
	Vitek 2								1		48	41		8	16
	Etest	5		1	4	25	54		1					0.5	1
CAS	CLSI-BMD				1	29	27	25	1	4	3			0.5	1
	Vitek 2					21	34	28		7				0.5	4
	Etest	9	1	9	22	33	5	4		7				0.25	2
VRC	CLSI-BMD		1	4	7	8	18	17	18	6	3	3	5	1	8
	Vitek 2			3	5	12	28	16	14	10	2			1	4
	Etest	1		3	2	8	15	36	12	3	7		3	1	16

[a] AMB, amphotericin B; CAS, caspofungin; VRC, voriconazole.

caused by this underreported pathogen in different geographic areas. In the past 5 years, *C. auris* fungemia has been reported from South Africa, South Korea, Japan, and India (3, 5–8). All of the reports from these countries confer the major issue of notable elevated MICs for azoles and caspofungin in *C. auris* and its misidentification by phenotypic methods. The present study employed MALDI-TOF MS, a more robust diagnostic technique, for rapid identification. The strength of the present study is that it developed a comprehensive reference database built with a large number of molecularly characterized *C. auris* strains from different geographical regions of India to supplement the Bruker Biotyper library, which has a database of only 3 strains from Korea and Japan. Not only was interspecies differentiation well characterized, but also the mass spectra variation at the intraspecies level separated *C. auris* isolates from India. It is pertinent to mention here that, previously, Indian *C. auris* isolates have been reported to exhibit differences in biochemical profiles compared to the Japanese and the Korean *C. auris* isolates (7, 8). Similarly, genotypic variation among *C. auris* isolates from different geographical regions has also been observed with M13 fingerprinting and amplified fragment length polymorphism analysis (7). It is evident from the present study that the high resolution and discriminatory power of MALDI-TOF MS facilitate differentiation of closely related cryptic species within the Metschnikowiaceae clade (23), which has also been documented previously for Mucorales, particularly the *Lichtheimia* species (24).

Another issue of concern is the misleading highly elevated MICs of AMB with Vitek automated readings in all *C. auris* isolates tested. The overall EA between Vitek automated readings and the CLSI-BMD method for AMB was very low (10%). Notably, the reference CLSI-BMD method in the present series showed reduced AMB MICs in 84% of *C. auris* isolates. Similarly, low AMB MICs (0.25 to 1 μg/ml) by CLSI-BMD were reported for 20 *C. auris* isolates from South Korea by Shin et al. (25). However, these authors observed a high EA (100%) between the CLSI-BMD and Vitek method for AMB, which is in contrast with the observations in the present study. This deviation could be attributed to the low number of isolates tested (25). Major errors of azole susceptibility in 218 isolates of 5 *Candida* species using another commercial automated reading system (ATB FUNGUS 3) have been reported recently from China, resulting in pseudohigh rates of antifungal resistance (26). In fact, the erroneously elevated MICs by the Vitek 2 automated reading method not only may lead to inappropriate selection of antifungal therapy but also depict false rates of high antifungal resistance in epidemiological studies. Further, the percentage of *C. auris* isolates that showed elevated CAS MICs (≥1 μg/ml) by CLSI-BMD in the present series (37%) declined to 12% using the Etest. The lower Etest MIC values than CLSI-BMD MIC values for CAS have also been reported earlier for other *Candida* species (27). Recently, the performance of the CAS Etest based on the recently revised CLSI breakpoints for *Candida* isolates showed that 13.1% were misclassified as intermediate or resistant (28). Also, marked interlaboratory variation has been observed with both CLSI-BMD and the EUCAST method for CAS susceptibility (29). In order to investigate the resistance mechanism with respect to elevated CAS MICs, in the present series we attempted to sequence *FKS* hot spot regions in *C. auris* isolates using known *FKS C. glabrata* primers due to a lack of published genomic data for *C. auris*. Although none of the isolates with elevated CAS MICs harbored mutations reported for echinocandin-resistant *C. glabrata*, the possibility of other mutations not reported so far could not be ruled out. Future studies on complete genomic analysis are warranted to detect true antifungal resistance in this significant pathogen.

Finally, *C. auris* is emerging as a serious multidrug nosocomial pathogen in many centers in India, which could be reliably and rapidly identified by MALDI-TOF MS. Notwithstanding the fact that routine laboratories heavily rely on commercial systems for identification and antifungal susceptibility testing for yeasts, a cautionary approach is recommended for isolates showing elevated MICs with these systems.

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